

AMENDMENTS TO THE SPECIFICATION

IN THE SPECIFICATION

On page 5, please amend the first and second paragraphs, starting on line 1 and ending on line 19, as follows:

C1
In one aspect, the invention provides purified or isolated nucleic acid comprising a sequence that encodes a peptide loop corresponding to amino acid residues 136-216 of wild-type IRP-2 from humans, wherein said sequence comprises a mutation in said peptide loop, wherein said mutation interferes with the ability of a cysteine residue present in said peptide loop to undergo oxidation. In one embodiment, the nucleic acid sequence can comprise at least one of ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15. Preferably, the nucleic acid sequence encodes a peptide comprising a sequence selected from the group consisting of ~~SEQ. ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ. ID NOS: 4, 6, 8, 10, 12, 14, and 16.

In another ~~preferred~~ preferred embodiment, the purified or isolated polypeptide comprises a peptide loop corresponding to amino acid residues 136-216 of wild-type IRP-2 from humans, wherein said sequence comprises a mutation in said peptide loop, wherein said mutation interferes with the ability of a cysteine residue present in said peptide loop to undergo oxidation. The IRP-2 protein can comprise a sequence selected from the group consisting of ~~SEQ. ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ. ID NOS: 4, 6, 8, 10, 12, 14, and 16. Preferably, the IRP-2 protein is selected from the group consisting of ~~SEQ. ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ. ID NOS: 4, 6, 8, 10, 12, 14, and 16. More preferably, the invention concerns the use of such a mutant polypeptide in a method of making a probe for the diagnosis of a neurodegenerative disease and involves generating an antibody that binds to an epitope present on said mutant polypeptide, wherein said antibody does not cross react with a wild-type IRP-2 protein or fragment thereof. The mutant can comprise a substitution or a deletion of a cysteine residue. Further, the generating step can comprise culturing cells which produce said antibody.

On page 6, please amend the first and second paragraphs, starting on line 5 and ending on line 13, as follows:

C2
In another ~~preferred~~ preferred embodiment, the invention concerns an antibody capable of specifically binding to a protein comprising an amino acid sequence selected from the group consisting of ~~SEQ. ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ. ID NOS: 4, 6, 8, 10, 12, 14, and 16. Preferably, the antibody specifically binds to a polypeptide comprising at least 10 consecutive

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amino acids of said protein and said protein has a mutation of a cysteine residue. More preferably, the antibody is a monoclonal antibody.

In another preferred embodiment, the invention concerns a purified or isolated antibody capable of specifically binding a mutant IRP-2 protein but does not specifically bind wild-type IRP-2 protein, wherein said mutant IRP-2 protein comprises a mutation in a peptide loop that corresponds to the amino acid sequence of ~~SEQ. ID. No. 2~~ SEQ. ID. NO: 2.

On page 8, please amend the second and third paragraphs, starting on line 9 and ending on line 28 as follows:

C3
Embodiments include nucleic acids encoding mutant IRP-2 proteins that are resistant to degradation in the body, complements thereto, and fragments of these proteins having at least one mutation. Desirably, these nucleic acids encode proteins that have mutations within a peptide loop corresponding to amino acid residues 136-216 of the sequence of human wild type IRP-2. A 189 nucleotide long fragment encoding a region of the wild type IRP-2 peptide loop is provided in the sequence listing. ~~(SEQ. ID. No. 1)~~ (SEQ ID NO:1). The full -length cDNA sequence encoding human wild type IRP-2 is provided in ~~SEQ. ID. No. 17~~ SEQ ID NO:17 and can be found in Guo et al., *J. Biol. Chem.* 270 16529 (1995), herein expressly incorporated by reference in its entirety. Additionally, the full -length cDNA sequence encoding rat wild type IRP-2 is provided in ~~SEQ. ID. No. 19~~ SEQ ID NO:19 and can be found in Guo et al., *J. Biol. Chem.* 270 16529 (1995), herein expressly incorporated by reference in its entirety. When reference is made to wild type IRP-2 nucleic acids, depending of the context, it is meant to refer to the wild type IRP-2 molecules including those provided in ~~SEQ. ID. Nos. 17~~ SEQ ID NOS:17 and/or 18 or that can be found in Guo et al., *J. Biol. Chem.* 270 16529 (1995), herein expressly incorporated by reference in its entirety.

Preferably, the nucleic acid embodiments have at least one mutation that results in an inability of a cysteine residue within the peptide loop corresponding to amino acid residues 136-216 of wild type human IRP-2 to undergo iron-dependent oxidation. This mutation may involve a substitution or deletion of a cysteine residue within this peptide loop or a mutation that perturbs the three-dimensional structure of the peptide loop so as to prevent iron-dependent oxidation. The sequences of several nucleic acids that encode a region of the peptide loop of a mutant IRP-2 protein are disclosed in ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15.

Please amend the next four paragraphs starting on page 8, line 29 and ending on page 10, line 6, as follows:

C4 Some nucleic acid embodiments are genomic DNA, RNA, and cDNA encoding a mutant IRP-2, a complement thereto or a fragment of these molecules that contain at least one mutation. Some embodiments comprise a plurality of mutations that result in multiple substitutions and/or deletions within this peptide loop (e.g., mutations that result in the substitution and/or deletion of more than one cysteine). Preferably, the nucleic acid embodiments include the nucleotide sequences shown in the sequence listing (~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ as SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15 and complements thereof and/or fragments thereof. Nucleic acid sequences encoding mutant IRP-2 from humans, mammals, and other organisms are also embodiments, as are methods for obtaining such sequences. The nucleic acid embodiments can be altered, mutated, or changed such that the alteration, mutation, or change results in a conservative amino acid replacement.

The polypeptide embodiments described herein concern mutant forms of IRP-2 that are resistant to degradation in the body and fragments of these proteins having at least one mutation. Desirably such polypeptides have a mutation in a peptide loop corresponding to amino acid residues 136-216 of human wild type IRP-2, which contributes to the stability of the molecule to degradation in the body (e.g., stability to proteosome degradation.) A 63 amino acid long peptide corresponding to a region of the wild type IRP-2 peptide loop is provided in the sequence listing. (~~SEQ. ID. No. 2~~) (SEQ. ID. NO:2) The full -length amino acid sequence of human wild type IRP-2 is provided in ~~SEQ. ID. No. 18~~ SEQ ID NO:18 can be found in Guo et al., *J. Biol. Chem.* 270 16529 (1995), herein expressly incorporated by reference in its entirety. Additionally, the full -length amino acid sequence of rat wild type IRP-2 is provided in ~~SEQ. ID. No. 18~~ SEQ. ID. NO:18 and can be found in Guo et al., *J. Biol. Chem.* 270 16529 (1995), herein expressly incorporated by reference in its entirety. When reference is made to wild type IRP-2 proteins, depending of the context, it is meant to refer to the wild type IRP-2 proteins including those provided in ~~SEQ. ID. Nos. 17~~ SEQ ID NOS:17 and/or 18 or that can be found in Guo et al., *J. Biol. Chem.* 270 16529 (1995), herein expressly incorporated by reference in its entirety.

Preferably, the polypeptide embodiments have at least one mutation that perturbs the iron-dependent oxidation of a cysteine residue within the peptide loop corresponding to amino acid residues 136-216 of human wild type IRP-2. This mutation may involve the substitution or deletion

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of a cysteine residue within this region or a mutation that perturbs the three-dimensional structure of the peptide loop so as to effect iron dependent oxidation of IRP-2. Some embodiments comprise a plurality of mutations within this peptide loop (e.g., more than one cysteine is mutated). Several mutant IRP-2 peptides are provided in ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16.

The polypeptide embodiments also include the partial or complete amino acid sequences shown in the sequence listing (~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16) and functional equivalents to such molecules including, but not limited to, the polypeptides ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16 having non-conservative amino acid substitutions and peptidomimetics that resemble these molecules. Additional embodiments include methods of preparing the polypeptides described herein and molecules that bind these polypeptides. Embodiments also include, for example, polyclonal and monoclonal antibodies that recognize wild-type and/or mutant IRP-2. Preferred antibodies bind to epitopes on mutant IRP-2 but not wild-type IRP-2 or vice versa so as to distinguish between these molecules. Novel approaches to manufacture the monoclonal and polyclonal antibodies described herein are provided.

Please amend the four paragraphs, starting on page 11, line 24 and ending on page 13, line 17, as follows:

The nucleotide sequences of the invention include, for example: (a) the DNA sequences shown in the sequence listing (~~SEQ ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ ID NOS: 3, 5, 7, 9, 11, 13, and 15); (b) nucleotide sequences encoding the amino acid sequences shown in the sequence listing (~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16); (c) any nucleotide sequence that hybridizes to the complement of the DNA sequences shown in the sequence listing (~~SEQ ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ ID NOS: 3, 5, 7, 9, 11, 13, and 15) under stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7.0% sodium dodecyl sulfate (SDS), 1 mM EDTA at 50°C and washing in 0.2 X SSC/0.2% SDS at 50°C; and (d) any nucleotide sequence that hybridizes to the complement of the DNA sequences that encode an amino acid sequence provided in the sequence listing (~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16) under less stringent conditions (e.g., hybridization in 0.5 M NaHPO₄, 7.0% sodium dodecyl sulfate (SDS), 1 mM EDTA at 37°C and washing in 0.2X SSC/0.2% SDS at 37°C).

Embodiments of the invention also include mutant IRP-2 nucleic acids that are isolated from other organisms (e.g., plants, molds, yeast, insects, animals, and mammals) whether naturally occurring or engineered. Approaches to isolate mutant IRP-2 nucleic acids in other species are provided *infra*. Embodiments also include fragments, modifications, derivatives, and variants of the sequences described above. Desired embodiments, for example, include nucleic acids having at least 9 consecutive bases unique to a mutant IRP-2 nucleic acid or a sequence complementary thereto and preferred fragments of the invention include at least 9 consecutive bases unique to a mutant IRP-2 nucleic acid or a sequence complementary thereto. In this regard, the nucleic acid embodiments can have from 9 to approximately 100 consecutive nucleotides. Some DNA fragments of the invention, for example, include nucleic acids having less than or equal to 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 125, 150, 175, 200, 225, and 240 consecutive nucleotides unique to a mutant IRP-2 nucleic acid and preferably encompass the region provided by the sequence of ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15 or a complement thereof. Preferably, the nucleic acid embodiments, however, comprise at least 12, 13, 14, 15, 16, 17, 18, or 19 consecutive nucleotides of a sequence unique to ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15 or complement thereof. More preferably, the nucleic acid embodiments comprise at least 20-30 consecutive nucleotides of a sequence unique to ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15 or complement thereof.

The nucleic acid embodiments can also be altered by mutation such as substitutions, additions, or deletions that provide for sequences encoding functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences that encode substantially the same mutant IRP-2 amino acid sequence as depicted in ~~SEQ. ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS: 4, 6, 8, 10, 12, 14, and 16 can be used in some embodiments. These include, but are not limited to, nucleic acid sequences comprising all or unique portions of a mutant IRP-2 nucleic acid or nucleic acids that complement all or unique parts of a mutant IRP-2 nucleic acid that has been altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change,

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or a functionally non-equivalent amino acid residue within the sequence, thus producing a detectable change.

The nucleic acid sequences described above have biotechnological and diagnostic use, e.g., in nucleic acid hybridization assays, Southern and Northern Blot analysis, etc. and the prognosis of neurodegenerative disease (e.g., Alzheimer's disease). By using the nucleic acid sequences disclosed in the sequence listing ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15, probes that complement wild type and/or mutant IRP-2 nucleic acids can be designed and manufactured by oligonucleotide synthesis. Desirable probes comprise a nucleic acid sequence that complements a nucleic acid sequence ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15 that is unique to these molecules as compared to ~~SEQ. ID No. 1~~ SEQ ID NO:1. These probes can be used to screen cDNA or genomic libraries from various organisms (e.g., plants, molds, fungi, yeast, insects, animals, and mammals) so as to isolate natural sources of the nucleic acid embodiments. Screening can be by filter hybridization, for example, using duplicate filters. The labeled probe preferably contains at least 15-30 base pairs of a nucleic acid sequence that complements a nucleic acid sequence ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15) that is unique to these molecules as compared to ~~SEQ. ID No. 1~~ SEQ ID NO:1. The hybridization washing conditions used are preferably of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence is originated.

On page 14, please amend the last paragraph, starting on line 30 and ending on page 15, line 9, as follows:

C6
The primers are selected to be substantially complementary to a portion of the nucleic acid sequence of ~~(SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15) that is unique to the mutant IRP-2 nucleic acid, thereby allowing the sequences between the primers to be amplified. Preferably, primers are 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30 nucleotides in length. The formation of stable hybrids depends on the melting temperature (T_m) of the DNA. The T_m depends on the length of the primer, the ionic strength of the solution and the G+C content. The higher the G+C content of the primer, the higher is the melting temperature because G:C pairs are held by three H bonds whereas A:T pairs have only two. The G+C content of the amplification primers of the present invention preferably ranges between 10 and 75 %, more preferably between 35 and 60 %, and most preferably between 40

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and 55 %. The appropriate length for primers under a particular set of assay conditions can be empirically determined by one of skill in the art.

Please amend the last paragraph on page 15, starting on line 25 and ending on page 16, line 2, as follows:

C7 Alternatively, a genomic library can be constructed using DNA obtained from an organism suspected of or known to carry the mutant IRP-2 allele, or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express the mutant IRP-2 allele. The normal IRP-2 gene or any suitable fragment thereof can then be labeled and used as a probe to identify the corresponding mutant IRP-2 allele in such libraries. Preferably, however, the probes complement a sequence of ~~SEQ. ID Nos. 3, 5, 7, 9, 11, 13, and 15~~ SEQ. ID NOS: 3, 5, 7, 9, 11, 13, and 15 that is unique to these mutant molecules. Clones containing the mutant IRP-2 gene sequences can then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

On page 18, please amend the two paragraphs, starting on line 1 and ending on page 19, line 4, as follows:

C8 The nucleic acids encoding a mutant IRP-2 protein or fragments thereof, described in the previous section, can be manipulated using conventional techniques in molecular biology so as to create recombinant constructs that express mutant IRP-2 protein or fragments of mutant IRP-2 protein. These polypeptides or derivatives thereof, include but are not limited to, those containing as a primary amino acid sequence all of the amino acid sequence substantially as depicted in the Sequence Listing (~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS: 4, 6, 8, 10, 12, 14, and 16) and fragments of ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS: 4, 6, 8, 10, 12, 14, and 16 at least three amino acids in length including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. Preferred fragments of a sequence of ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS: 4, 6, 8, 10, 12, 14, and 16 are at least three amino acids and comprise amino acid sequence unique to mutant IRP-2 proteins including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. The mutant IRP-2 peptide fragments can be, for example, less than or equal to 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69,

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70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, and 100 amino acids in length so long as said peptide has an amino acid that is unique to a mutant IRP-2 peptide, as compared to ~~SEQ ID No. 2~~ SEQ ID NO:2..

Embodiments of the invention encompass proteins that are functionally equivalent to the mutant IRP-2 proteins encoded by the nucleotide sequences described in ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16, as judged by any of a number of criteria, including but not limited to the inability to be oxidized, the inability to be ubiquitinated, and the ability to remain stable to proteosome degradation. Such functionally equivalent mutant IRP-2 proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the mutant IRP-2 nucleotide sequences described above but, which result in a silent change, thus producing a functionally equivalent gene product. For example, embodiments include mutant IRP-2 proteins that have one or more amino acid residues within the mutant IRP-2 polypeptide of ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16 and fragments of ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16 that are substituted by another amino acid of a similar polarity that acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence can be selected from other members of the class to which the amino acid belongs. For example, the non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. The aromatic amino acids include phenylalanine, tryptophan, and tyrosine.

On page 19, please amend the last paragraph starting on line 23 and ending on page 20, line 8, as follows:

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In several embodiments, mutant IRP-2 proteins and fragments of mutant IRP-2 proteins are expressed in a cell line. For example, some cells are made to express the IRP-2 polypeptide of in ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16 or fragments of in ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14, and 16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16. The sequences, constructs, vectors, clones, and other materials comprising these embodiments can advantageously be in enriched or isolated form. As used herein, "enriched" means that the

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concentration of the material is at least about 2, 5, 10, 100, or 1000 times its natural concentration (for example), advantageously 0.01%, by weight, preferably at least about 0.1% by weight. Enriched preparations from about 0.5%, 1%, 5%, 10%, and 20% by weight are also contemplated. The term "isolated" requires that the material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide present in a living animal is not isolated, but the same polynucleotide, separated from some or all of the coexisting materials in the natural system, is isolated. It is also advantageous that the sequences be in purified form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition. Isolated proteins have been conventionally purified to electrophoretic homogeneity by Coomassie staining, for example. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

Please amend the last paragraph on page 23, starting on line 24 and ending on page 24, line 7, as follows:

CTD

The mutant IRP-2 gene products or fragments thereof can also be expressed in plants, insects, and animals so as to create a transgenic organism. Plants and insects of almost any species can be made to express these molecules. Desirable transgenic plant systems having a wild type or mutant IRP-2 or fragment thereof include, for example, ~~Arabidopsis~~ Arabidopsis, maize, and ~~ehlamydomonas~~ chlamydomonas. Desirable insect systems having a wild type or mutant IRP-2 or fragment thereof include, for example, ~~D. melanogaster and C. elegans~~ D. melanogaster and C. elegans. Animals of any species, including, but not limited to, amphibians, reptiles, birds, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, dogs, cats, and non-human primates, e.g., baboons, monkeys, and chimpanzees can be used to generate a mutant IRP-2 transgenic animals. Transgenic organisms desirably exhibit germline transfer of mutant IRP-2 proteins or fragments thereof. Some transgenic organisms exhibit complete knockouts or point mutations of one or more existing IRP-2 genes. For example, in one embodiment, a transgenic animal comprises at least one point mutation at a cysteine residue within the peptide loop of IRP-2 corresponding to amino acid residues 136-216 and preferably within the region provided in ~~SEQ. ID. No. 2~~ SEQ ID NO:2. The most preferred transgenic animal embodiments have

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mutations that resemble the mutant IRP-2 fragments provided in ~~SEQ ID Nos. 4, 6, 8, 10, 12, 14,~~
and ~~16~~ SEQ ID NOS:4, 6, 8, 10, 12, 14, and 16.

Please amend the first paragraph on page 26, starting on line 1 and ending on line 16, as follows:

C11
In general, the design and synthesis of a peptidomimetic involves starting with the amino acid sequence of the peptide and conformational data (e.g., geometry data, such as bond lengths and angles) of a desired peptide (e.g., the most probable simulated peptide). That data is then used to determine the geometries that should be designed into the peptidomimetic. Numerous methods and techniques are known in the art for performing this step, any of which could be used. (See, e.g., Farmer, P. S., Drug Design, (Ariens, E. J. ed.), Vol. 10, pp. 119-143 (Academic Press, New York, London, Toronto, Sydney and San Francisco) (1980); Farmer, et al., in TIPS, 9/82, pp. 362-365; Verber et al., in TINS, 9/85, pp. 392-396; Kaltenbronn et al., in *J. Med. Chem.* 33: 838-845 (1990); and Spatola, A. F., in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, Vol. 7, pp. 267-357, Chapter 5, "Peptide Backbone Modifications: A Structure-Activity Analysis of Peptides Containing Amide Bond Surrogates. Conformational Constraints, and Relations" (B. Weisten, ed.; Marcell Dekker: New York, pub.) (1983); Kemp, D. S., "Peptidomimetics and the Template Approach to Nucleation of β -sheets β -sheets and α -helices in Peptides," Tibeck, Vol. 8, pp. 249-255 (1990). Additional teachings can be found in U.S. Patent Nos. 5,288,707; 5,552,534; 5,811,515; 5,817,626; 5,817,879; 5,821,231; and 5,874,529. The section below describes the preparation and use of antibodies directed to wild type or mutant IRP-2 proteins or fragments thereof.

Please amend the last paragraph starting on page 29, line 22 and ending on page 30, line 4, as follows:

C12
Some functional assays involve binding assays that utilize multimeric agents. One form of multimeric agent concerns a manufacture comprising a wild type or mutant IRP-2 protein or fragment thereof disposed on a support. These multimeric agents provide the wild type or mutant IRP-2 protein or fragment thereof in such a form or in such a way that a sufficient affinity is achieved. A multimeric agent having a n wild type or mutant IRP-2 protein or fragment thereof is obtained by joining the desired polypeptide to a macromolecular support. A "support" can be a termed a carrier, a protein, a resin, a cell membrane, or any macromolecular structure

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C12
used to join or immobilize such molecules. Solid supports include, but are not limited to, the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, animal cells, ~~Duracyte®~~ DURACYTE®, artificial cells, and others. A wild type or mutant IRP-2 protein or fragment thereof can also be joined to inorganic carriers, such as silicon oxide material (e.g., silica gel, zeolite, diatomaceous earth or aminated glass) by, for example, a covalent linkage through a hydroxy, carboxy or amino group and a reactive group on the carrier.

Please amend the two paragraphs starting on page 31, line 27 and ending on page 32, line 15 as follows:

C13
In one characterization assay, for example, the ability of mutant support-bound IRP-2 peptides to undergo oxidation and ubiquitination is compared with the ability of wild type support-bound IRP-2 peptides to undergo oxidation and ubiquitination. By one method, oxidation of support bound IRP-2 is performed at the concentration of ~~0.1 µg/µl~~ 0.1 µg/µl protein in a ~~20 µl~~ 20 µl reaction mixture (25mM Hepes-NaOH, pH 7.2 and 40mM KCl) in the presence of ~~50 µM~~ 50 µM FeCl₃ and 10mM DTT at 37°C for 15-30 minutes. In some embodiments, it is desired to use Tris-carboxyethyl-phosphine (TCEP) at 1mM to reduce the disulfides instead of DTT. In particular, when reduced IPR-2 is desired, preferably, TCEP at 1mM is used in a reaction mixture without iron for 15-30 minutes at 37°C.

Once the oxidized and/or reduced IRP-2 supports are made, an *in vitro* ubiquitination assay can be performed as follows. The oxidized and/or reduced support-bound wild type and mutant IRP-2 is added to ~~400 µg~~ 400 µg RD4 S100 lysates, 5mM ~~MgCl₂~~ MgCl₂, 2mM ATP, 2mM DTT, ~~6 µg~~ 6 µg ubiquitin, 25mM Tris-Cl (pH 7.6) and 60mM KCl for 5 minutes. Reactions are stopped by adding ice cold buffer containing 1% NP-40, 0.5% deoxycholate, 50mM Tris-Cl (pH8.0), 150mM NaCl, and 0.1% SDS. The support bound conjugate is washed in this buffer three times; the beads are spun down at 1500xg between washes. The beads are boiled for 10 minutes in 2X Laemmli buffer and are separated on a suitable SDS PAGE (e.g., 6%-15%). The separated proteins are transferred to a membrane by electroblotting and the presence of ubiquitin can be verified by Western blotting with an affinity purified polyclonal or monoclonal anti-ubiquitin antibody. This assay will verify the ability of oxidized and reduced forms of mutant and wild type IRP-2 to interact with ubiquitin.

Please amend page 33, first complete paragraph starting on line 5 and ending on line 18, as follows:

C14
Additionally, a cell based characterization assay can be performed. For example, COS cells can be transfected to express mutant and/or wild type IRP-2 proteins. (See e.g., Samaniego et al., *J. Biol. Chem.* 269:30904 (1994), herein expressly incorporated by reference in its entirety, for a protocol for transfecting COS cells to express wild type IRP-2). After selecting transformants, aliquots of the positive expressing cells are placed under oxidative stress. By one approach, oxidative stress is brought about by raising the concentration of ferric ammonium citrate in the medium to 400 ~~µg/ml~~ 400 µg/ml. By another approach, exposure to oxidative stress is accomplished in a serum- and phenol red-free medium containing 0.1 mM H₂O₂ for 30 min. The cells are collected immediately or are cultured in H₂O₂ or iron-free medium to allow them to recover from oxidative stress. Control cells are treated exactly as the exposed cells except that H₂O₂ or iron are not included in the medium. The viability of the cells after exposure to H₂O₂ or iron can be monitored by exclusion of trypan blue and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide staining. The levels of reduced glutathione can also be determined. Further, the levels of ATP in the cells can be monitored using the bioluminescent somatic cell assay kit (Sigma) according to the manufacturer's instructions.

Please amend the second paragraph on page 38, starting on line 13 and ending on line 28 as follows:

C15
Any ~~adressable~~ addressable array technology known in the art can be employed with this aspect of the invention. One particular embodiment of polynucleotide arrays is known as ~~Genechips™~~ GENECHIPS™, and has been generally described in US Patent 5,143,854; PCT publications WO 90/15070 and 92/10092. These arrays are generally produced using mechanical synthesis methods or light directed synthesis methods, which incorporate a combination of photolithographic methods and solid phase oligonucleotide synthesis. (Fodor et al., *Science*, 251:767-777, (1991)). The immobilization of arrays of oligonucleotides on solid supports has been rendered possible by the development of a technology generally identified as "Very Large Scale Immobilized Polymer Synthesis" (VLSPIS™) in which, typically, probes are immobilized in a high density array on a solid surface of a chip. Examples of VLSPIS™ technologies are provided in US Patents 5,143,854 and 5,412,087 and in PCT Publications WO 90/15070, WO 92/10092 and WO 95/11995, which describe methods for forming oligonucleotide arrays through

CS techniques such as light-directed synthesis techniques. In designing strategies aimed at providing arrays of nucleotides immobilized on solid supports, further presentation strategies were developed to order and display the oligonucleotide arrays on the chips in an attempt to maximize hybridization patterns and diagnostic information. Examples of such presentation strategies are disclosed in PCT Publications WO 94/12305, WO 94/11530, WO 97/29212, and WO 97/31256.

Please amend the second paragraph on page 49, starting on line 3 and ending on line 16 as follows:

CS Another preferred embodiment concerns image analysis performed for each mice on a single slice immediately anterior to the slice where the hippocampus can be seen curling inferiorly. This position corresponded approximately to bregma -3.60 mm and maximized the cross-sectional area of each region of interest (ROI). Cheshire™ CHESHIRE™ image processing software (Hayden Image Processing Group, Waltham, MA) is used to outline and analyze the ROI's that are confirmed by a second researcher. The bilateral ROI's included the amygdala (and associated nuclei), piriform cortex (including part of the entorhinal and perirhinal cortices), hippocampus, retrosplenial cortex (including motor and somatosensory cortices) and thalamus. A two pixel width separates the hippocampi and retrosplenial ROI's. A line is drawn across the bottom of both hippocampi that extends across the cortex demarcated the inferior border of the retrosplenial ROI. The piriform and amygdala ROI's are abutted each other and extended the same distance superiorly and inferiorly. Medially two to four pixels separate the thalamus from the amygdaloid ROI, to minimize signal contribution from the lateral ventricle. A 5 by 5 pixel square is centered within the thalamus. For the 3D gradient echo images, information from slices around this slice is evaluated.

On page 50, last paragraph, starting on line 16 and ending on line 28, please amend the paragraph as follows:

C17 For immunocytochemical labeling of frozen tissue sections of the mice, tissue is fixed as described above for the apoptosis assay (Green *et al.* 1995; Green *et al.* 2001). The fixed tissue is labeled with primary antibodies (anti-Irp-1, anti-Irp-2, anti-ferritin, anti-transferrin/transferrin receptor, +~~amyloid~~ β -amyloid, ubiquitin and hemosiderin). Incubation with primary antibodies/antisera is 16 hrs at 4°C, followed by washing in PBS containing 0.05% Tween-20 (PBST). Antibodies that are not directly conjugated to a fluorescent molecule are secondarily labeled with alexa-488, alexa-594, Cy-2 or Cy-5 anti-mouse or rabbit IgG antibodies

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C17
for a minimum incubation period of 4 hrs at 25°C. Just prior to completion of the secondary antibody incubation, DAPI (1µg/ml) or propidium iodide (PI, 5µg/ml), depending on the ability to dual label and/or confocal microscopy as the analytical endpoint, is added for 10 min. Excess secondary antibody and nuclear counterstain is removed by washing in PBST, and the tissue is protected with permafluor and glass coverslips and is dried flat in the dark prior to quantitative analysis (LSC and Confocal microscopy as described below) and photography. Non-specific fluorescence is determined by incubating control sections with non-immune sera and secondary antibody, or secondary antibody alone.

Please amend the two paragraphs starting on page 57, line 21 and ending on page 58, line 21 as follows:

C18
Antibodies specific for oxidized and reduced forms of wild type and mutant IRP-2 peptides were prepared as follows. Seven clones having one or more cysteine residues in the peptide loop of amino acid residues 138-216 of IRP-2 substituted with alanine were created by conventional techniques in molecular biology. The "C1A" clone has a substitution of the first cysteine proximal to the N-terminus with an alanine. (~~SEQ. ID. No. 4~~) (SEQ ID NO:4). The "C2A" clone has a substitution of the second cysteine proximal to the N-terminus with an alanine. (~~SEQ. ID. No. 6~~) (SEQ ID NO:6). The "C3A" clone has a substitution of the third cysteine proximal to the N-terminus with an alanine. (~~SEQ. ID. No. 8~~) (SEQ ID NO:8). The "C12A" clone has substitutions of the first and second cysteines proximal to the N-terminus with an alanine(~~SEQ. ID. No. 10~~) (SEQ ID NO:10). The "C23A" clone has substitutions of the second and third cysteines proximal to the N-terminus with an alanine. (~~SEQ. ID. No. 12~~) (SEQ ID NO:12). The "C13A" clone has substitutions of the first and third cysteines proximal to the N-terminus with an alanine. (~~SEQ. ID. No. 14~~) (SEQ ID NO:14). The "C123A" clone has substitutions of the first, second, and third cysteines proximal to the N-terminus with an alanine. (~~SEQ. ID. No. 16~~) (SEQ ID NO:16). A wild type peptide sequence was also produced recombinantly in ~~E. Coli~~ *E. Coli* (~~SEQ. ID. No. 2~~) (SEQ ID NO:2).

Once the recombinant peptides were isolated, they were either oxidized or reduced. Oxidation of IRP-2 was performed at the concentration of ~~0.1µg/µl~~ 0.1µg/µl protein in a ~~20:1~~ 20µl reaction mixture (25mM Hepes-NaOH, pH 7.2 and 40mM KCl) in the presence of ~~50µM~~ 50µM FeCl₃ and 10mM DTT at 37°C for 15-30 minutes. The reduced forms of the peptides were obtained by incubating the peptide in Tris-carboxyethyl-phosphine (TCEP) at 1mM for 15-

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C18
30 minutes at 37°C. Once the oxidized and reduced peptides were obtained, they were coupled with KLH and were used to generate antibodies in mice. Hybridomas were made using conventional methods and the clones were screened for the production of antibodies specific for the particular peptide used to inoculate the mouse. The antibody generated to the wild type peptide was found to recognize both the peptide of ~~SEQ. ID. No. 2~~ SEQ ID NO:2 and full-length IRP-2 in both ELISA and Western blot. A 1:5000 dilution was found sufficient. Another selection process was also used to screen some of the antibodies. Because the oxidation of IRP-2 can depend on the conversion of a cysteine residue to aminomalonic acid, an IRP-2 peptide having aminomalonic acid was synthesized. The clones were screened for reactivity to the aminomalonic acid peptide and also the native IRP-2 peptide. Clones that were reactive to the aminomalonic peptide but not the native peptide were selected. By using the teachings described in this example, antibodies to both mutant and wildtype IRP-2 proteins can be made. These antibodies can be used in the diagnostic assays described herein to identify a subject's predilection to a neurodegenerative disease. The next example describes a similar approach that was used to make an antibody specific for wild-type IRP-2.

Please amend the paragraphs starting with the second complete paragraph on page 58, line 28 and ending on page 59, line 14 as follows:

C19
Competitive binding assays were then conducted between 4G11 and the other 5 clones, to determine whether they recognized the same or different epitopes. Only one (14F7) did not significantly inhibit the binding of 4G11, and is assumed to bind at a different site. Thus, 14F7 and 4G11-HRP became the basis for the capture ELISA assay (described below), which can also be used for detection of IRP-2 in biological samples. The assay is sensitive down to ~~1 µg/mL~~ 1 µg/mL and shows excellent linearity.

The capture assay was performed as follows. Unlabeled antibody was diluted in carbonate buffer, pH 9.6 (Sigma #C-3041), usually to 1-10 µg/mL. The individual antibody concentration may need to be determined empirically, starting with 10 µg/mL and working downward. It is important not hinder antigen binding by overcrowding and the lowest concentration that will still give a strong signal was selected. The antibodies were then plated, approx. ~~100 µL~~ 100 µL per well, in ~~Immulon-1~~ IMMULON-1 plates (Dynex #3355), covered with tape (Falcon #3073), and incubated overnight at 4°C.

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Subsequently, the plates were warmed to room temp. and the wells were washed 3X with PBS (w/o tween) (Cellgro, #20-031-CV, 10X Concentrate diluted to 1X.) The plates were then blocked with SuperBlock (Pierce #37515), by adding ~~200 µL~~ 200 µL to each well, emptying by inversion, and repeating the process for a total of 3X. The wells were then washed 3X with PBS-Tween (PBS + 0.05% Tween-20, Sigma #P-6585). Diluent (control), antigen, and standards (approx. ~~100 µL~~ 100 µL) were added to the wells. The diluent used was carrier (10% SuperBlock in PBS-Tween). The wells were taped and the reaction was allowed to take place for 1 hr at room temp. (Shaking the plate will greatly increase sensitivity in assays). Subsequently, the wells were washed 3X with PBS-Tween.

Next, approx. ~~100 µL~~ 100 µL of an HRP-tagged detection antibody diluted in carrier was added. (The manufacturer's recommendations was followed when a commercial product was used and the appropriate dilution of home grown antibodies was empirically determined.) The wells were taped and the reaction was allowed to take place for 1 hr at room temp. (Shaking the plate will greatly increase sensitivity in assays). Subsequently, the wells were washed 3X with PBS-Tween. Then approx. 100 uL substrate (Bio-Rad #172-1067) was added to the wells. The reaction was allowed to take place uncovered for approx. 30 min. and readings at an absorbance at 630 nm, ref. 490 nm were taken. Assay parameters were such that an O.D. of around 2.0 was obtained in about 30 min. incubation for the most concentrated antigen samples in the assay. When scouting out appropriate dilutions of capture antibody, antigen, and detection antibody, it may be helpful to do a "checkerboard" assay. The example below describes an approach that was used to join antiIRP-2 antibodies to beads.

Please amend the first paragraph on page 63, starting on line 1 and ending on line 6, as follows:

The relative expression of functional transferrin receptors on cells from test subjects is determined by flow cytometric analysis. Briefly, isolated mononuclear cells are stained with 100 ng of phycoerythrin-conjugated human ~~transferin~~ transferrin (BioE Inc.) for 15 minutes and washed once with PBS to remove unbound conjugate prior to flow cytometric analysis. Expression of functional receptors (that is, receptors actually capable of binding transferrin) is directly proportional to the intensity of fluorescence of the cells.

Please amend the third paragraph on page 63, starting on line 20 and ending on line 24, as follows:

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C20 Briefly, one million mononuclear cells activated by ~~ActiCyte-TC~~ ACTICYTE-TC are washed with PBS and stained with the phosphatidylserine binding protein Annexin-V-FITC (200 ng, Caltag, South San Francisco, CA) and the DNA-intercalating dye Propidium Iodide (4 ug). Cells that are positive for Annexin-V alone or Annexin-V and Propidium Iodide are considered as early or late-stage apoptotic, respectively, while cells that are positive for Propidium iodide alone are considered necrotic.
